Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Campylobacter spp. subtype analysis using gel-based repetitive extragenic palindromic-PCR discriminates in parallel fashion to *flaA* short variable region DNA sequence analysis

K.L. Hiett, B.S. Seal and G.R. Siragusa

U.S. Department of Agriculture, Agricultural Research Service, Poultry Microbiological Safety Research Unit, Russell Research Center, Athens, GA, USA

Keywords
Campylobacter, disease surveillance, food safety microbiology, genotyping, poultry, repetitive extragenic palindromic-PCR, zoonoses.

Correspondence
Kelli L. Hiett, U.S. Department of Agriculture, Agricultural Research Service, Poultry Microbiological Safety Research Unit, Russell Research Center, P.O. Box 5677, Athens, GA 30604-5677, USA.
E-mail: khiett@saa.ars.usda.gov

2005/1212: received 11 October 2005, revised 20 March 2006 and accepted 24 March 2006

doi:10.1111/j.1365-2672.2006.03026.x

Abstract

Aims: The repetitive extragenic palindromic-PCR (rep-PCR) subtyping technique, which targets repetitive extragenic DNA sequences in a PCR, was optimized for *Campylobacter* spp. These data were then used for comparison with the established genotyping method of *flaA* short variable region (SVR) DNA sequence analysis as a tool for molecular epidemiology.
Methods and Results: Uprime Dt, Uprime B1 or Uprime RI primers were utilized to generate gel-based fingerprints from a set of 50 *Campylobacter* spp. isolates recovered from a variety of epidemiological backgrounds and sources. Analysis and phenogram tree construction, using the unweighted pair group method with arithmetic mean, of the generated fingerprints demonstrated that the Uprime Dt primers were effective in providing reproducible patterns (100% typability, 99% reproducibility) and at placing isolates into epidemiological relevant groups. Genetic stability of the rep-PCR Uprime Dt patterns under nonselective, short-term transfer conditions revealed a Pearson's correlation approaching 99%. These same 50 *Campylobacter* spp. isolates were analysed by *flaA* SVR DNA sequence analysis to obtain phylogenetic relationships.
Conclusions: The Uprime Dt primer-generated rep-PCR phenogram was compared with a phenogram generated from *flaA* SVR DNA sequence analysis of the same isolates. Comparison of the two sets of resulting genomic relationships revealed that both methods segregated isolates into similar groups.
Significance and Impact of the Study: These results indicate that rep-PCR analysis performed using the Mo Bio Ultra Clean Microbial Genomic DNA Isolation Kit for DNA isolation and the Uprime DT primer set for amplification is a useful and effective tool for accurate differentiation of *Campylobacter* spp. for subtyping and epidemiological analyses.

Introduction

Campylobacter spp. are Gram-negative, microaerophilic bacteria, presently considered to be the leading bacterial aetiological agent of acute gastroenteritis in the human population (Mead *et al.* 1999). The total number of *Campylobacter* spp. enteritis cases in the United States is estimated as 2·4 million annually, or approximately 1–2% of the population per year (Blaser and Reller 1981; Slutsker *et al.* 1998; Friedman *et al.* 2000; Oberhelman and Taylor 2000), with a similar incidence level in Europe (Adak *et al.* 2005; van Duynhoven *et al.* 2005; Meldrum *et al.* 2005). *Campylobacter* spp. are commonly found in the intestines and caeca of many agriculturally important animals but are primarily associated with poultry (Newell and Fearnley 2003). It is widely presumed that human

infection occurs as a result of consuming insufficiently cooked meat or by cross-contamination to precooked food or processed food that requires no further preparation (Park *et al.* 1981; Kinde *et al.* 1983; Bryan and Doyle 1995). Handling and consumption of poultry and poultry-related products account for up to 75% of all *Campylobacter* spp. infections (Griffiths and Park 1990). The high colonization incidence of poultry and the resultant clinical infections in humans have prompted a number of investigations focused upon identifying and subsequently eliminating sources of *Campylobacter* spp. contamination in chickens.

In order to facilitate source tracking, practical subtyping techniques that provide accurate, reproducible and adequate discriminatory capability are critical. A primary requirement for adopting a subtyping methodology is the capacity to classify subtypes in an appropriate epidemiological context. Upon developing and applying a new typing method to an organism, the method should initially be tested on isolates from a known epidemiological background to assess the discriminatory power (Barrett *et al.* 2004; Riley 2004).

Other factors that should be considered upon adopting a new subtyping technology include the ability of the method to discriminate clonality, simplicity in performing the technique, throughput and cost. A number of subtyping technologies for differentiating *Campylobacter* spp. have been evaluated (On 1996; Wassenaar and Newell 2000) and include restriction fragment length polymorphism (RFLP) (Owen *et al.* 1985), PCR-RFLP (Iriarte and Owen 1996), *flaA* typing (Alm *et al.* 1993; Nachamkin *et al.* 1993), *flaA* gene short variable region (SVR) DNA sequence analysis (Meinersmann *et al.* 1997), pulse-field gel electrophoresis (PFGE; Chang and Taylor 1990; Yan *et al.* 1991), and amplified fragment length polymorphism (AFLP; Duim *et al.* 1999). However, the lack of a universal nomenclature system for the resulting isolate profiles continues to be a hindrance to efficient molecular epidemiology.

Repetitive extragenic palindromic-PCR (rep-PCR) is an amplification-based method that targets known, conserved, repetitive DNA sequences that are usually present in bacterial genomes as multiple copies (Versalovic *et al.* 1991; Lupski and Weinstock 1992). Initially, DNA is isolated from the organism of interest. Single or multiple defined primers are then utilized for PCR under high stringency conditions. The targeted sequences are generally spaced 20–400 bp apart throughout the genome and are rarely located within open reading frames, hence the term extragenic. The resulting amplicons are resolved and analysed using computer algorithms. Examples of repetitive DNA motifs include rep, enterobacterial repetitive intergenic consensus (ERIC) elements, BOX elements and RepMP3 (Stern *et al.* 1984; Wenzel and Herrmann 1988; Hulton *et al.* 1991; Martin *et al.* 1992). In the present investigation, we evaluated rep-PCR technology for typing *Campylobacter jejuni* and *Campylobacter coli* from a variety of samples and epidemiological backgrounds. Genomic stability of the rep-PCR Dt patterns was determined under nonselective, short-term transfer conditions. Additionally, the rep-PCR technique was compared with the well-established technique of *flaA* SVR DNA sequence analysis for subtype analysis of *C. jejuni*.

Materials and methods

Bacterial isolates and growth conditions

A total of 48 *Campylobacter* spp. isolates were analysed in an epidemiological context during this study and are reported in Table 1. Forty-six of the isolates tested were *C. jejuni*. One isolate was *C. coli* and one isolate was determined to be a *Campylobacter* spp. other than *jejuni* or *coli*. Two isolates were included in duplicate as a blind test of the subtyping technology. Twenty-four of the isolates originated from an epidemiological survey of poultry production and processing facilities conducted in the United States (Hiett *et al.* 2002). Ten, six, and eight of these isolates originated from separate investigations conducted in Arkansas, California, and Georgia, respectively. The remaining 24 isolates were collected from an epidemiological survey conducted in Iceland (Stern *et al.* 2003). *Campylobacter jejuni* isolate ATCC 49943 was used for rep-PCR Uprime Dt pattern stability investigations. All *Campylobacter* spp. isolates were grown on Brucella FBP agar and incubated at 42°C for 36–48 h in microaerobic atmosphere (5% O_2, 10% CO_2 and 85% N_2) as described previously (Stern *et al.* 1992).

Rep-PCR analyses

Template DNA was purified, using either the QIAamp DNA Mini kit (Qiagen, Inc., Valencia, CA, USA) or the Mo Bio Ultra Clean Microbial Genomic DNA Isolation Kit (Mo Bio Laboratories, Inc., Solana Beach, CA, USA), from a lawn of bacteria representing a single colony of each isolate. DNA was isolated in duplicate for each isolate. Additionally, fingerprints were generated in duplicate for each isolate from the same template DNA preparation (100 ng). Thus, a total of four rep-PCR reactions were performed on each isolate in order to ensure consistency. Repetitive element-PCR was conducted (Versalovic *et al.* 1991) using three independent primer sets (Uprime Dt, Uprime B1 and Uprime RI primer sets), buffers and reagents in the rep_{pro} DNA Fingerprinting Kit (Bacterial Barcodes, Houston, TX, USA) with AmpliTaq polymerase

Journal compilation © 2006 The Society for Applied Microbiology, Journal of Applied Microbiology **101** (2006) 1249–1258

Table 1 Isolates of *Campylobacter* spp. utilized for rep-PCR and *flaA* gene DNA sequence analyses comparison

Project	Isolate number	Subproject	Source	Additional information
USA Epi	C01	Arkansas	Chicken	AL01 04/98 faecal dropping
	C17	Arkansas	Chicken	AL18 04/98 faecal dropping
	C05	Arkansas	Chicken	AL21 04/98 faecal dropping
	C46	Arkansas	Chicken	ALP07 04/98 carcass rinse
	C09	Arkansas	Chicken	ALP18 04/98 carcass rinse
	C03	Arkansas	Chicken	ALP20 04/98 carcass rinse
	C13	Arkansas	Mouse	AL56 04/98 intestine
	C38	Arkansas	Wild bird	AL62 04/98 faeces
	C50	Arkansas	Fly	AL66 04/98 rinse
	C02	Arkansas	Pretransport crate	ALP48 04/98 swab
	C49	California	Chicken	CL01 04/99 faecal dropping
	C47	California	Chicken	CL08 04/99 faecal dropping
	C29	California	Chicken	CL25 04/99 faecal dropping
	C19	California	Production facility	CL49 drag swab
	C41	California	Post-transport crate	CLP57 swab
	C24	California	Post-transport crate	CLP64 swab
	C07	California	Post-transport crate	CLP64 swab
	C30	Georgia	Chicken	GH02 04/98 faecal dropping
	C35	Georgia	Chicken	GHP10 04/98 carcass rinse
	C26	Georgia	Chicken	GHP18 04/98 carcass rinse
	C25	Georgia	Chicken	GHP24 04/98 carcass rinse
	C36	Georgia	Chicken	GH79 04/98 caecal dropping
	C11	Georgia	Mouse	GH56 04/98 intestine
	C43	Georgia	Wild bird	GH59 04/98 faeces
	C12	Georgia	Pretransport crate	GHP47 04/98 swab
Iceland Epi	C04	August	Chicken	0002 A1 08/09/1999 carcass rinse
	C34	August	Chicken	0033 A1 08/09/1999 carcass rinse
	C28	August	Chicken	0061 A1 08/09/1999 carcass rinse
	C45	August	Chicken	0075 A1 08/09/1999 carcass rinse
	C08	September	Chicken	1506 A1 09/28/1999 carcass rinse
	C10	September	Chicken	1581 A1 09/28/1999 carcass rinse
	C31	September	Chicken	1591 A1 09/28/1999 carcass rinse
	C21	October	Chicken	1841 A1 10/11/1999 carcass rinse
	C42	October	Chicken	1841 A1 10/11/1999 carcass rinse
	C48	October	Chicken	1951 A1 10/12/1999 carcass rinse
	C15	October	Chicken	2001 A1 10/18/1999 carcass rinse
	C06	August	Human	H001 08/11/1999 faeces
	C22	August	Human	H005 08/20/1999 faeces
	C37	October	Human	H051 10/14/1999 faeces
	C44	October	Human	H053 10/22/1999 faeces
	C20	October	Human	H058 10/27/1999 faeces
	C39	October	Human	H059 10/27/1999 faeces
	C32	October	Human	H060 10/27/1999 faeces
	C27	November	Human	H062 11/01/1999 faeces
	C16		Calf	3283-ES52799 faeces
	C23		Duck	3299–58899 faeces
	C40		Duck	3314–61999 faeces
	C18		Hog	3297–57699A faeces
	C33		Hog	3309–61099A faeces
	C14		Turkey	3280–437992 faeces

(Perkin-Elmer, Foster City, CA, USA). An initial thermocycle reaction was completed with a 2-min denaturing at 95°C, 1 min annealing at 40°C and an 8 min extension at 65°C. This was followed by 31 cycles of denaturing at 94°C for 3 s and 92°C for 30 s, 1 min annealing at 40°C and an 8 min extension at 65°C. Amplification products were resolved in 1·5% agarose TAE cooled gels. Gel images were captured using a computerized video image

system (EpiChem3 Darkroom Gel Documentation System, UVP, Upland, CA, USA) and analysed using Bionumerics software (Applied Maths BVBA, Austin, TX, USA). Molecular weight standards (1 kb ladder) were included in each gel to allow normalization of gel images for valid between-gel comparisons of fingerprints. Fingerprints were analysed quantitatively as analog densitometric scans of gel tracks, which were digitally compared with each other in a pairwise fashion using Pearson's correlation coefficient (band optimization 1·0%). Intensity differences in individual bands, caused by the absence or presence of additional priming sites, are interpreted as band differences, and thus as distinct fingerprints. Observed differences in overall fingerprint intensities are not interpreted as banding differences. Similarity matrices were constructed and phenograms were then inferred from the resulting similarity matrices according to the unweighted pair group method with averaging (UPGMA).

Both positive and negative controls were included with each set of rep-PCR reactions performed. For the positive controls, a previously characterized colony of *C. jejuni* was used, while negative controls contained sterile water as the template for rep-PCR.

DNA sequence analyses of the *flaA* SVR

Isolated colonies of *Campylobacter* spp. were suspended in 300 μl of sterile H_2O and placed in a heat block set to 100°C for 10 min. Ten microlitres of each boiled cell suspension were utilized as template for *flaA* SVR PCR with the following primers: FLA242FU: 5′-CTA TGG ATG AGC AAT TWA AAA T-3′ and FLA625RU: 5′-CAA GWC CTG TTC CWA CTG AAG-3′ (Meinersmann *et al.* 1997). A 35-cycle reaction was used with 1 min denaturing at 96°C, 1 min annealing at 52°C and a 1 min extension at 72°C. The resulting product was approximately 425 bp. Automated DNA sequence (Smith *et al.* 1986) was generated using either the FLA242FU primer or the FLA625RU primer with the Big-Dye Dye-Terminator Cycle Sequencing Kit (ABI-PE, Foster City, CA, USA). Data were assembled with Sequencher 4.2 (GeneCodes Corp., Ann Arbor, MI, USA) and aligned using ClustalX (Thompson *et al.* 1994). Aligned sequences were compared and phenograms generated using the UPGMA algorithm with HKY85 distance measurements in PAUP*4·0 (Swofford 1998).

Discriminatory index (1-D) for segregating isolates

Simpson's index of diversity (Hunter and Gaston 1988) was calculated to determine the relative discriminatory powers of rep-PCR and *flaA* SVR DNA sequence analysis for the collection of isolates. Similarity values of 90% and 98% were used to distinguish between rep-PCR and *flaA* SVR subtypes, respectively. When performing the calculations, only one representative isolate from the same epidemiological background that grouped within the same cluster was used.

Results

Rep-PCR optimization

In an effort to determine optimal parameters for rep-PCR analysis of *Campylobacter* spp., initial investigations were conducted employing two genomic DNA isolation kits (QIAamp DNA Mini Kit and Mo Bio Ultra Clean Microbial Genomic DNA Isolation Kit) coupled with three primer sets, Uprime Dt, Uprime B1 and Uprime RI, for subsequent amplification. Both DNA isolation kits yielded template DNA that amplified with the Uprime Dt primer set only (data not shown). Visual inspection of the banding patterns obtained using both DNA isolation procedures revealed that DNA templates produced with the Mo Bio Ultra Clean Microbial Genomic DNA Isolation Kit resulted in more defined banding patterns. Therefore, the Mo Bio DNA isolation Kit was employed for all subsequent investigations. Two distinct DNA preparations were made from each isolate. Uprime Dt fingerprints were generated in duplicate for each isolate using the same template DNA preparation providing for a total of four repeat rep-PCR reactions for each isolate; all duplicates possessed virtually identical banding patterns with Pearson's correlations approaching 99%. On average, four to eight bands were produced ranging in size from 400 to 5000 bp (Fig. 1). Informative rep-PCR patterns were obtained from 100% of the *Campylobacter* spp. isolates tested using the Uprime Dt primers.

Rep-PCR Uprime Dt pattern stability and rep-PCR analysis of isolates

Campylobacter jejuni ATCC 49943 was passaged, on non-selective media, three times over a 5-day period. Five individual colonies from each of the three passages were selected and used to test rep-PCR Uprime Dt pattern stability. All fingerprints generated from the passed isolates were identical (data not shown). Consequently, the discriminatory power of the rep-PCR Uprime Dt analysis was determined using a library of *Campylobacter* spp. isolates with distinct epidemiological backgrounds. Overall rep-PCR Uprime Dt analysis placed isolates in epidemiological relevant groups (Fig. 1). Isolates obtained from Icelandic poultry operations and Icelandic human clinical cases were generally segregated closely together with the exceptions of isolates C21, C32 and C42. Two of these

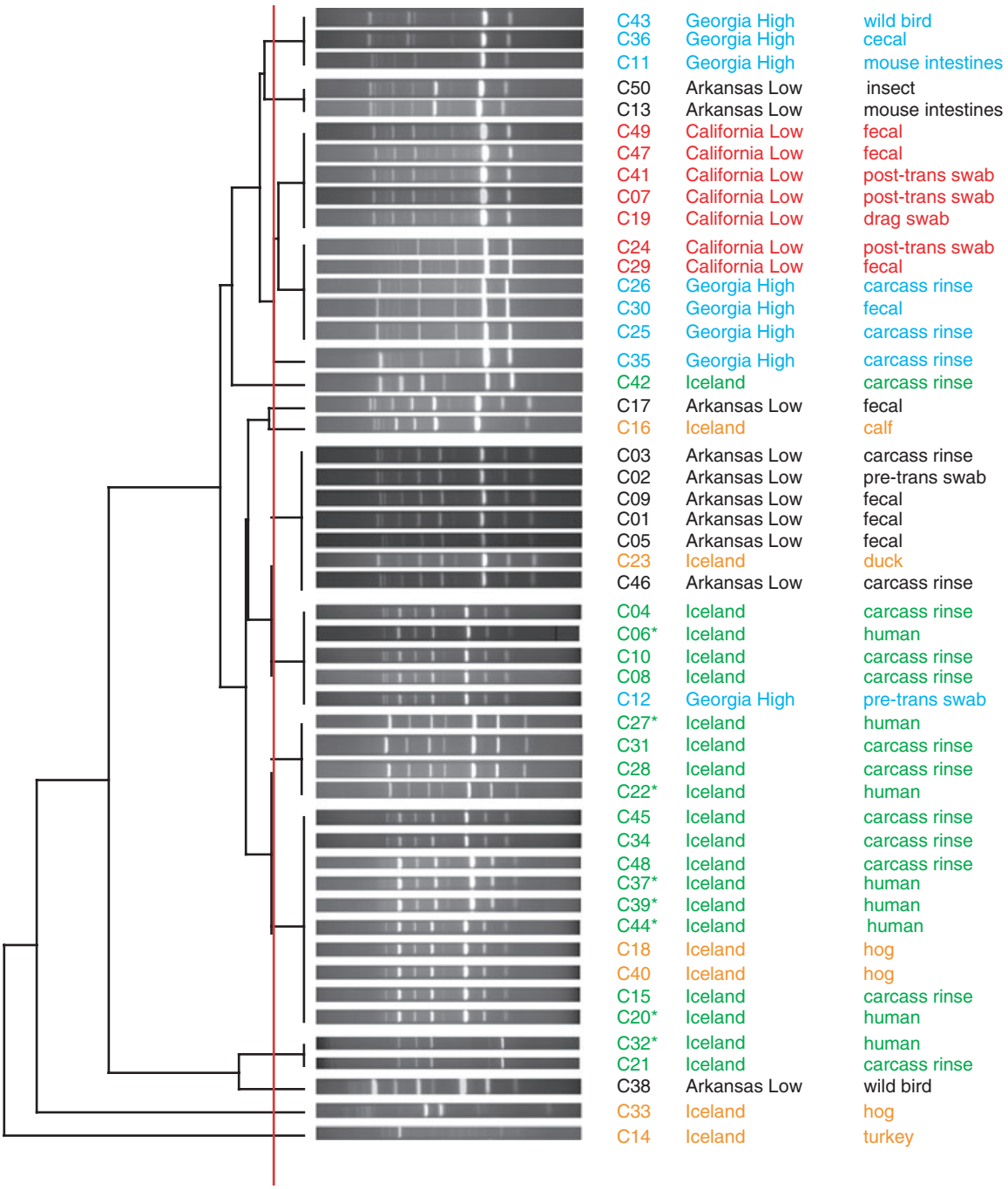

Figure 1 Cluster analyses of rep-PCR patterns from *Campylobacter* spp. isolates. Patterns for rep-PCR were generated by amplification with Uprime Dt primers followed by resolution of the products using gel electrophoresis. Relationships were determined based on gel images in the Bionumerics software. Additionally, four replicate rep-PCRs for each isolate were used to infer relationships. Intensity differences in individual bands, caused by the absence or presence of additional priming sites, are interpreted as band differences, and thus as distinct fingerprints. Observed differences in overall fingerprint intensities are not interpreted as banding differences. Branches were collapsed to the level of 90% similarity for clarity. Bacterial isolate designations are as reported in Table 1.

isolates, C21 and C32, separated together but independently of all other Iceland-associated isolates. The remaining isolate, C42, segregated independently of all other Iceland-associated isolates. Of the six isolates obtained from other agricultural animals in Iceland, two isolates recovered from hogs, C18 and C40, grouped together

along with Icelandic poultry and human-associated isolates. The remaining four isolates, C14, C16, C23 and C33, obtained from a turkey, calf, duck and hog, respectively, separated independently. Isolates C14 and C33 grouped distantly from all other isolates in this investigation. Interestingly, isolate C14 was determined to be a *Campylobacter* species other than *jejuni* or *coli*, while isolate C33 was determined to be a *C. coli*.

Comparison of *Campylobacter* spp. isolates obtained during an Arkansas (USA) epidemiological investigation of a poultry farm revealed the presence of multiple rep-PCR subtypes within the flock and on the carcasses (Fig. 1). In general, isolates recovered from faecal samples during production were closely related (≥90% similarity) to isolates recovered from the processed carcasses. Isolates C17 and C46, obtained from a poultry faecal sample and from a carcass rinse sample, respectively, were exceptions. These two isolates grouped independently of the other Arkansas poultry-associated isolates. The three environmentally associated isolates recovered during the Arkansas study, C13, C38 and C50 (mouse, wild bird and insect, respectively), separated distantly from the other Arkansas isolates.

Analysis of *Campylobacter* spp. isolates obtained during an epidemiological survey of poultry conducted in Georgia (USA) again revealed the presence of multiple rep-PCR subtypes within the flock (Fig. 1). A *Campylobacter* spp. isolate, C30, recovered from a faecal sample during production separated closely (≥90% similarity) with isolates recovered from the processed carcasses of the same flock. The second rep-PCR subtype observed during the production of the Georgia flock, isolate C36, grouped closely to an isolate recovered from the faeces of a wild bird, isolate C43. An isolate obtained from a transport crate prior to movement of the flock, C12, grouped distantly from all other isolates obtained during the Georgia investigation. Interestingly, this isolate was closely related to isolates obtained from poultry and from humans during the Iceland investigations. Analysis of *Campylobacter* spp. isolates obtained during an epidemiological survey of poultry conducted in California (USA) revealed two dominate clusters of isolates that segregated together.

Analysis of *flaA* SVR sequences from *Campylobacter* spp. isolates

Subtype analysis of the *Campylobacter* spp. library using *flaA* SVR DNA sequence analysis placed isolates in epidemiological relevant groups (Fig. 2). Isolates obtained from Icelandic poultry operations and Icelandic human clinical cases generally segregated closely together. Isolates C21, C32 and C42 were exceptions in that these isolates were identical, but clustered distantly from the other Iceland isolates. The six isolates recovered from agricultural animals other than poultry grouped distantly from the poultry and human isolates. Three of the nonpoultry isolates, C16, C18, and C40, grouped with poultry production and processing related isolates from Arkansas (USA).

The *flaA* SVR DNA sequence analysis of the *Campylobacter* spp. isolates obtained during the Arkansas epidemiological investigation placed all isolates recovered from poultry faeces and from carcass rinses in the same clade (Fig. 2). However, the three environmentally associated isolates recovered during the Arkansas study, C13, C38 and C50 (mouse, wild bird and insect, respectively), separated distantly from the other Arkansas isolates. Analysis of *Campylobacter* spp. isolates obtained during an epidemiological survey of poultry conducted in Georgia (USA) placed all of the isolates, with the exception of C12 (pre-transport crate swab), in the same cluster. Isolate C12 separated with Arkansas poultry faecal and carcass rinse sample isolates along with three of the isolates from other agricultural animals in Iceland. All isolates recovered during the California (USA) investigation had identical *flaA* SVR DNA sequences and clustered with poultry-associated isolates from Georgia.

Discriminatory indices of the analyses

The discriminatory indices (DIs) of rep-PCR and *flaA* SVR DNA sequence analyses were determined at 90% and 98% similarity values, respectively. The DI for rep-PCR subtype analysis of these isolates was 0·8364, while the DI for *flaA* SVR DNA sequence subtype analysis was 0·9394.

Discussion

A variety of DNA-based typing methods (reviewed by Olive and Bean 1999) have been developed and optimized for the differentiation of bacteria during molecular epidemiological investigations. Examples of technologies regularly utilized for the differentiation of *Campylobacter* spp. include PFGE (Gibson *et al.* 1995), PCR-RFLP (Ayling *et al.* 1996), ribotyping (Fitzgerald *et al.* 1996), randomly amplified polymorphic DNA (Mazurier *et al.* 1992), AFLP (Duim *et al.* 1999), DNA sequence analysis of specific genes, including *flaA* (Meinersmann *et al.* 1997), and multilocus sequence typing (MLST; Manning *et al.* 2003; Sails *et al.* 2003; Dingle *et al.* 2005; Miller *et al.* 2005).

Rep-PCR is an amplification-based method that targets known, conserved, repetitive DNA sequences usually present in bacterial genomes in multiple copies (Versalovic *et al.* 1991; Lupski and Weinstock 1992). ERIC sequences were identified in *Escherichia coli* and *Salmonella typhimurium* (Sharples and Lloyd 1990; Hulton *et al.* 1991), while a second repetitive element, the BOX sequence, was

Journal compilation © 2006 The Society for Applied Microbiology, Journal of Applied Microbiology **101** (2006) 1249–1258

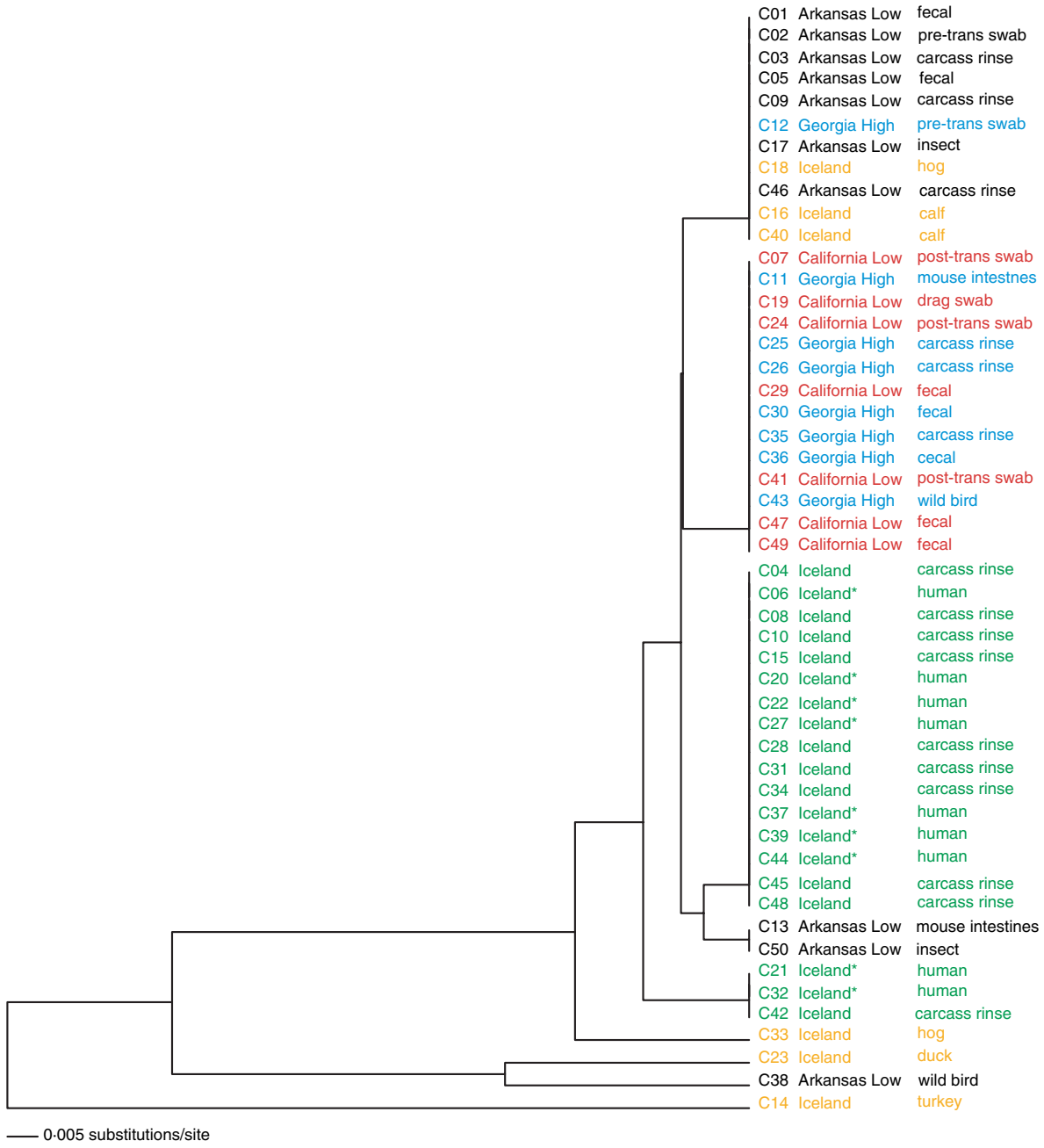

Figure 2 Phylogenetic relationships based on *flaA* short variable region (SVR) gene sequence. Amplification and DNA sequence analysis of the *flaA* gene SVR were as described in section Materials and methods. The *flaA* SVR region sequences were aligned and analysed in PAUP* to generate the dendrogram.

identified in *Streptococcus pneumoniae* (Martin *et al.* 1992; Koeuth *et al.* 1995). rep-PCR analysis was previously utilized to obtain strain-specific patterns of various bacteria (Versalovic *et al.* 1995); therefore, we examined the potential of this technology for the temporal and regional discrimination of *Campylobacter* spp. isolates. The relationships obtained from the application of the rep-PCR method were compared with those obtained from *flaA* SVR DNA sequence analysis (Meinersmann *et al.* 1997). To our knowledge, this is the first report wherein rep-PCR was utilized for typing *Campylobacter* spp. isolates with well-defined epidemiological histories.

Our results demonstrated that overall, rep-PCR, using the Mo Bio Ultra Clean Microbial Genomic DNA Isolation

Kit for DNA isolation and the Uprime DT primer set for amplification, generated fingerprints for *Campylobacter* spp. that were highly reproducible and informative for all isolates examined during this study. Additionally, isolates were segregated into spatially and temporally epidemiological relevant groups. These findings are in agreement with other reports for bacteria recovered from poultry sources (Amonsin *et al.* 2002) and for closely related bacterial species (Burucoa *et al.* 1999). Furthermore, the *Campylobacter* spp. isolates examined during these investigations were passaged in the laboratory (short term) to determine whether gel patterns obtained by rep-PCR were stable. Short-term multiple passages appeared to have no adverse affects on reproducibility of the rep-PCR gel patterns. These findings were somewhat surprising, given the observation that repeat sequences are lacking from the two *C. jejuni* genomes currently published (Parkhill *et al.* 2000; Fouts *et al.* 2005).

Overall, rep-PCR possessed a discriminatory power less than that from *flaA* SVR DNA sequence analysis of the *Campylobacter* spp. isolates examined during this study. For example, isolate C14, recovered from a turkey in Iceland, was the phylogenetic outlier utilizing both techniques and C33, recovered from a hog in Iceland, segregated essentially independently. Although techniques such as MLST (Manning *et al.* 2003) or PFGE (Gibson *et al.* 1995) may be the principle methods relied upon for typing bacterial isolates, they are expensive and time-consuming relative to rep-PCR. Herein it has been reported that rep-PCR can be utilized to reliably subtype *Campylobacter* spp. Recently, rep-PCR technology has advanced to the point of eliminating agarose slab gels by employing microfluidic devices for resolution of fluorescently labelled amplicons (Healy *et al.* 2005). The newest iteration of the method potentially eliminates gel-to-gel variation, thereby minimizing lab-to-lab variation and maximizing reproducibility. Additionally, the new format permits on-line data collection for analyses, thus allowing for pattern input into databases and bioinformatics.

Acknowledgements

This research was supported by ARS, USDA CRIS project#6612-32000-034-00D. Appreciation is extended to Ms Johnna Garrish and Ms Robin Kuntz for excellent technical assistance.

References

Adak, G.K., Meakins, S.M., Yip, H., Lopman, B.A. and O'Brien, S.J. (2005) Disease risks from foods, England and Wales, 1996–2000. *Emerg Infect Dis* **11**, 365–372.

Alm, R.A., Guerry, P. and Trust, T.J. (1993) Distribution and polymorphism of the flagellin genes from isolates of *Campylobacter coli* and *Campylobacter jejuni*. *J Bacteriol* **175**, 3051–3057.

Amonsin, A., Wellehan, J.F., Li, L.L., Laber, J. and Kapur, V. (2002) DNA fingerprinting of *Pasteurella multocida* recovered from avian sources. *J Clin Microbiol* **40**, 3025–3031.

Ayling, R.D., Woodward, M.J., Evans, S. and Newell, D.G. (1996) Restriction fragment length polymorphism of polymerase chain reaction products applied to the differentiation of poultry campylobacters for epidemiological investigations. *Res Vet Sci* **60**, 168–172.

Barrett, T.J., Ribot, E. and Swaminathan, B. (2004) Molecular subtyping for epidemiology: issues in comparability of patterns and interpretation of data. In *Molecular Microbiology: Diagnostic Principles and Practice* ed. Persing, D.H., Tenover, F.C., Versalovic, J., Tang, Y.-W., Unger, E.R., Relman, D.A. and White, T.J. pp. 259–268. Washington, DC: American Society for Microbiology.

Blaser, M.J. and Reller, L.B. (1981) *Campylobacter enteritis*. *New Engl J Med* **305**, 1444–1452.

Bryan, F.L. and Doyle, M.P. (1995) Health risks and consequences of *Salmonella* and *Campylobacter jejuni* in raw poultry. *J Food Prot* **58**, 326–344.

Burucoa, C., Lhomme, V. and Fauchere, J.L. (1999) Performance criteria of DNA fingerprinting methods for typing *Helicobacter pylori* isolates: experimental results and meta-analysis. *J Clin Microbiol* **37**, 4071–4080.

Chang, N. and Taylor, D.E. (1990) Use of pulsed-field agarose gel electrophoresis to size genomes of *Campylobacter* species and to construct a SalI map of *Campylobacter jejuni* UA580. *J Bacteriol* **172**, 5211–5217.

Dingle, K.E., Colles, F.M., Falush, D. and Maiden, M.C. (2005) Sequence typing and comparison of population biology of *Campylobacter coli* and *Campylobacter jejuni*. *J Clin Microbiol* **43**, 340–347.

Duim, B., Wassenaar, T.M., Rigter, A. and Wagenaar, J. (1999) High-resolution genotyping of *Campylobacter* strains isolated from poultry and humans with amplified fragment length polymorphism fingerprinting. *Appl Environ Microbiol* **65**, 2369–2375.

van Duynhoven, Y.T., de Jager, C.M., Kortbeek, L.M., Vennema, H., Koopmans, M.P., van Leusden, F., van der Poel, W.H., van den Broek, M.J. *et al.* (2005) A one-year study of outbreaks of gastroenteritis in The Netherlands. *Epidemiol Infect* **133**, 9–21.

Fitzgerald, C., Owen, R.J. and Stanley, J. (1996) Comprehensive ribotyping scheme for heat-labile serotypes of *Campylobacter jejuni*. *J Clin Microbiol* **34**, 265–269.

Fouts, D.E., Mongodin, E.F., Mandrell, R.F., Miller, W.G., Rasko, D.A., Ravel, J., Brinkac, L.M., DeBoy, R.T. *et al.* (2005) Major structural differences and novel potential virulence mechanisms from the genomes of multiple *Campylobacter* species. *PLoS Biol* **3**, e15.

Friedman, C.R., Neimann, J., Wegener, H.C. and Tauxe, R.V. (2000) Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialized nations. In *Campylobacter*, 2nd edn ed. Nachamkin, I. and Blaser, M.J. pp. 121–138. Washington, DC: American Society for Microbiology.

Gibson, J.R., Fitzgerald, C. and Owen, R.J. (1995) Comparison of PFGE, ribotyping, and phage-typing in the epidemiologic analysis of *Campylobacter jejuni* serotype HS2 infections. *Epidemiol Infect* **115**, 215–225.

Griffiths, P.L. and Park, R.W.A. (1990) Campylobacters associated with human diarrhoel disease. *J Appl Bacteriol* **69**, 281–301.

Healy, M., Huong, J., Bittner, T., Lising, M., Frye, S., Raza, S., Schrock, R., Manry, J. *et al.* (2005) Microbial DNA typing by automated repetitive-sequenced-based PCR. *J Clin Microbiol* **43**, 199–207.

Hiett, K.L., Stern, N.J., Fedorka-Cray, P., Cox, N.A., Musgrove, M.T. and Ladely, S. (2002) Molecular subtype analyses of *Campylobacter* spp. from Arkansas and California poultry operations. *Appl Environ Microbiol* **68**, 6220–6236.

Hulton, C.S.J., Higgins, C.F. and Sharp, P.M. (1991) ERIC sequences: a novel family of repetitive elements in the genomes of *Escherichia coli, Salmonella typhimurium*, and other enterobacteria. *Mol Microbiol* **142**, 1881–1893.

Hunter, P.R. and Gaston, M.A. (1988) Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *J Clin Microbiol* **26**, 2465–2466.

Iriarte, P. and Owen, R.J. (1996) PCR-RFLP analysis of the large subunit (23S) ribosomal RNA genes of *Campylobacter jejuni*. *Lett Appl Microbiol* **23**, 163–166.

Kinde, H., Genigeorgis, C.A. and Pappaioanou, M. (1983) Prevalence of *Campylobacter jejuni* in chicken wings. *Appl Environ Microbiol* **45**, 1116–1118.

Koeuth, T., Versalovich, J. and Lupski, J.R. (1995) Differential subsequence conservation of interspersed repetitive *Streptococcus pneumoniae* BOX elements in diverse bacteria. *Genome Res* **5**, 408–418.

Lupski, J.R. and Weinstock, G.M. (1992) Short, interspersed repetitive DNA sequences in prokaryotic genomes. *J Bacteriol* **174**, 4525–4529.

Manning, G., Dowson, C.G., Bagnall, M.C., Ahmed, I.H., West, M. and Newell, D.G. (2003) Multilocus sequence typing for comparison of veterinary and human isolates of *Campylobacter jejuni*. *J Clin Microbiol* **69**, 6370–6379.

Martin, B., Humbert, O., Camara, M., Guenzi, E., Walker, J., Mitchell, T., Andrew, P., Prudhomme, M. *et al.* (1992) A highly conserved repeated DNA element located in the chromosome of *Streptococcus pneumoniae*. *Nucleic Acids Res* **20**, 3479–3483.

Mazurier, S., van de Giessen, A., Heuvelman, K. and Wernars, K. (1992) RAPD analysis of *Campylobacter* isolates: DNA fingerprinting without the need to purify DNA. *Lett Appl Microbiol* **14**, 260–262.

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffen, P.M. and Tauxe, R.V. (1999) Food related illness and death in the United States. *Emerg Infect Dis* **5**, 607–625.

Meinersmann, R.J., Helsel, L.O., Fields, P.I. and Hiett, K.L. (1997) Discrimination of *Campylobacter jejuni* isolates by *fla* gene sequencing. *J Clin Microbiol* **33**, 2810–2814.

Meldrum, R.J., Griffiths, J.K., Smith, R.M. and Evans, M.R. (2005) The seasonality of human campylobacter infection and *Campylobacter* isolates from fresh, retail chicken in Wales. *Epidemiol Infect* **133**, 49–52.

Miller, W.G., On, S.L., Wang, G., Fontanoz, S., Lastovica, A.J. and Mandrell, R.E. (2005) Extended multilocus sequence typing system for *Campylobacter coli*, *C. lari*, *C. upsaliensis*, and *C. helveticus*. *J Clin Microbiol* **43**, 2315–2329.

Nachamkin, I., Bohachik, K. and Patton, C.M. (1993) Flagellin gene typing of *Campylobacter jejuni* by restriction fragment length polymorphism analysis. *J Clin Microbiol* **31**, 1531–1536.

Newell, D. and Fearnley, C. (2003) Sources of *Campylobacter* colonization in broiler chickens. *Appl Environ Microbiol* **69**, 4343–4351.

Oberhelman, R.A. and Taylor, D.N. (2000) *Campylobacter* infections in developing countries. In *Campylobacter*, 2nd edn ed. Nachamkin, I. and Blaser, M.J. pp. 139–154. Washington, DC: American Society for Microbiology.

Olive, D.M. and Bean, P. (1999) Principles and applications of methods for DNA-based typing of microbial organisms. *J Clin Microbiol* **37**, 1661–1669.

On, S.L.W. (1996) Identification methods for campylobacters, helicobacters, and related organisms. *Clin Microbiol Rev* **9**, 405–422.

Owen, R.J., Beck, A. and Borman, P. (1985) Restriction endonuclease digest patterns of chromosomal DNA from nitrate-negative *Campylobacter jejuni*-like organisms. *Eur J Epidemiol* **1**, 281–287.

Park, C.E., Stankiewicz, Z.K., Lovett, J. and Hunt, J. (1981) Incidence of *Campylobacter jejuni* in fresh eviscerated whole market chickens. *Can J Microbiol* **27**, 841–842.

Parkhill, J., Wren, B.W., Mungall, K., Ketley, J.M., Churcher, C., Basham, D., Chillingworth, T., Davies, R.M. *et al.* (2000) The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* **403**, 665–668.

Riley, L.W. (2004) Principles and approaches. In *Molecular Epidemiology of Infectious Diseases: Principles and Practices*, 1st edn ed. Riley, L.W. pp. 1–28, Washington, DC: American Society of Microbiology.

Sails, A.D., Swaminathan, B. and Fields, P.I. (2003) Clonal complexes of *Campylobacter jejuni* identified by multilocus sequence typing correlate with strain associations identified by multilocus enzyme electrophoresis. *J Clin Microbiol* **41**, 4058–4067.

Sharples, G.J. and Lloyd, R.G. (1990) A novel repeated DNA sequence located in the intergenic regions of bacterial chromosomes. *Nucleic Acids Res* **18**, 6503–6508.

Slutsker, L., Altekruse, S.F. and Swerdlow, D.L. (1998) Foodborne diseases: emerging pathogens and trends. *Infect Dis Clin North Am* **12**, 199–216.

Smith, L.M., Sanders, J.Z., Kaiser, R.J., Hughs, P., Dodd, C., Connell, C.R., Heines, C., Kent, S.B.H. *et al.* (1986) Fluorescence detection in automated DNA sequence analysis. *Nature* **321**, 673–681.

Stern, M.J., Ames, G.F.L., Smith, N.H., Robinson, E.C. and Higgins, C.F. (1984) Repetitive extragenic palindromic sequences: a major component of the bacterial genome. *Cell* **37**, 1015–1026.

Stern, N., Wojton, B. and Kwiatek, K. (1992) A differential-selective medium and dry ice-generated atmosphere for recovery of *Campylobacter jejuni*. *J Food Prot* **55**, 514–517.

Stern, N.J., Hiett, K.L., Alfredsson, G.A., Cox, N.A., Kristinsson, K.G., Reiersen, J., Briem, H., Gunnarsson, E. *et al.* (2003) *Campylobacter* spp. in Icelandic poultry operations and human disease. *Epidemiol Infect* **130**, 23–32.

Swofford, D. (1998) *PAUP*4.0: Phylogenetic Analysis Using Parsimony*. Sunderland, MA: Sinauer Associates, Inc.

Thompson, J., Higgins, D. and Gibson, T. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties, and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Versalovic, J., Koeuth, T. and Lupski, J.R. (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res* **19**, 6823–6831.

Versalovic, J., Kapur, V., Koeuth, T., Mazurek, G.H., Whittam, T.S., Musser, J.M. and Lupski, J.R. (1995) DNA fingerprinting of pathogenic bacteria by fluorophore-enhanced repetitive sequenced-based polymerase chain reaction. *Arch Pathol Lab Med* **119**, 23–29.

Wassenaar, T.M. and Newell, D.G. (2000) Genotyping of *Campylobacter* spp. *Appl Environ Microbiol* **66**, 1–9.

Wenzel, R. and Herrmann, R. (1988) Repetitive DNA sequences in *Mycoplasma pneumoniae*. *Nucleic Acids Res* **16**, 8337–8350.

Yan, W., Chang, N. and Taylor, D.E. (1991) Pulsed-field gel electrophoresis of *Campylobacter jejuni* and *Campylobacter coli* genomic DNA and its epidemiologic application. *J Infect Dis* **163**, 1068–1072.